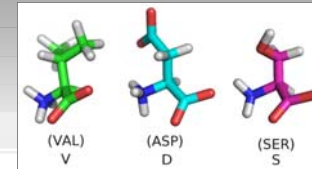


Possible Novel Inhibitor Identified for Protein Tyrosine Phosphatase 1B (PTP1B) Using Virtual Screening

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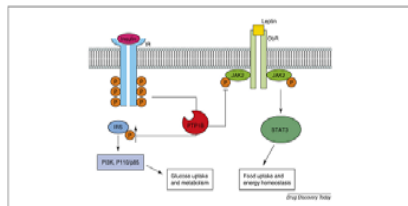


ABSTRACT

PTP1B functions as a negative or positive modulator in numerous signal transduction pathways—however, the focus of this research was only on the negative pathway of PTP1B. Over-activation of PTP1B weakens the insulin signal, resulting in insulin resistance, and so reducing the activity of PTP1B would be expected to strengthen insulin sensitivity. For this reason, PTP1B is a powerful target for the treatment of type 2 diabetes, which is a disorder characterized by high blood glucose in the context of insulin resistance. Virtual drug screening, an *in silico* evaluation of large libraries of chemical structures, serves as an excellent starting point for drug discovery to identify those structures that would most likely bind to a drug target. This technique has the ability to process thousands of potential enzyme – ligand complexes in a fast, cost-efficient way. In the wet lab, PTP1B DNA was cloned into pNIC-Bsa4 as a preparation to express it as a protein. The two vectors were mixed for annealing, and the transformation step using competent cells ultimately yielded in successfully cloned DNA. For protein expression, the cloned DNA was introduced into another type of competent cell – BL21(DE3) – for protein translation. The expressed protein was then purified and ready for enzyme assay. PTP1B inhibition assay was performed with three different compounds that were the best ligand determined by the virtual screening step. Of those three, the assay indicated that the “cocktail” (combination of compound 6703891 and inhibitor #5) compound displayed most inhibition on the PTP1B enzyme activity. Since this cocktail showed significant inhibition, further experimentation would be necessary to determine which inhibited or if it was a product of both. Once this is completed, the successful inhibitor (if any) would be tested at lower and lower concentrations to determine if it has an IC₅₀ (amount of inhibitor necessary to inhibit half of enzyme) that is low enough to be used as a drug.

INTRODUCTION

Our target enzyme, PTP1B, has the ability to catalyze protein tyrosine dephosphorylation. The negative pathway of PTP1B associates with and dephosphorylates activated insulin receptor (IR) or insulin receptor substrates (IRS). PTP1B can also interact with leptin signaling, which is related to development of obesity.



Over-activation of PTP1B weakens the insulin signal, resulting in insulin resistance, and so reducing the activity of PTP1B would be expected to strengthen insulin sensitivity. For this reason, PTP1B is a powerful target for the treatment of type 2 diabetes, which is a disorder characterized by high blood glucose in the context of insulin resistance.

There has been plenty of research over PTP1b over the last decade and a lot of research articles and reviews are already available. But still, even though years of active research has resulted in discovery of many types of inhibitors, an inhibitor that is fit for commercial use has not been developed yet.

In order to discover possible novel inhibitor for the PTP1B enzyme, virtual screening, a method that makes possible to find out how well each ligand binds to the target protein *in silico*, was used. Such technique enabled us to screen through thousands of compounds in a relatively short time in a cost efficient way.

MATERIALS & METHODS

Virtual Screening Obtained 3-Dimensional structure of PTP1B enzyme from the Protein Data Base and used GOLD for the *in silico* docking process to Screen thousands of compounds using libraries from Cambridge and Maybridge. Screened compounds and ranked according to their GOLD fitness score. Viewed top ranked compounds using PyMol and purchased compounds after considering the Lipinski's Rule of 5.

DNA Cloning/PCR amplification was performed for PTP1B coding sequence from a cloning vector obtained from the Harvard/ASU Protein Structure Initiative (PSI) using the BioRad PCR thermocycler. Annealed the amplified PTP1B into pNIC-Bsa4 using ligation independent cloning (pNIC-Bsa4 vector has 6x His tag for protein purification, kanamycin resistance and T7 promoter for protein expression). Annealed plasmids were transformed into One Shot MAX Efficiency DH5α T1B Competent Cells (NEB, Woods Hollow, MA) competent cells. PTP1B gene was isolated and concentrated from the competent cells with Mini-prep (NEB, Woods Hollow, MA).

Protein Expression Mini-prepped sample was transformed into another competent cells, BL21(DE3) (Invitrogen). A colony from transformation was first added to small culture (5ml LB broth), then to a large culture (250 ml LB broth). Then, its OD (600nm) was taken and once it reached ~0.8, IPTG, Isopropyl β-D-1-thiogalactopyranoside, was added to induce the protein expression of T7 polymerase. 5-prime chromatography column in Ni-NTA affinity purification was performed to purify the protein. Ni-NTA can bind strongly to the His-tagged PTP1B protein and filter out other undesired proteins. Elution buffer with high concentration of imidazole was used to elute the His-tagged protein from the Ni-NTA resin.

Enzyme Assay The purchased compounds were dissolved in 50% DMSO. Assay was done with two control groups, one with no PTP1B enzyme and one with no inhibitor. All inhibitor assays were run with varying inhibitor concentrations ranging from 0.1mM to 1000mM. 400mg of enzyme was added to each tube (except for the control) and each tube had PNPP concentration of 5mM with a final volume of 410ul. The absorbance assay is possible through the cleavage of PNPP by the enzyme into an absorbing species. The absorbance value was taken at 410nm wavelength. Afterwards, in order to verify whether the DMSO had any inhibition effect to the enzyme, assay with DMSO (without the enzyme) was performed with DMSO concentration ranging from 0% to 5% of the final volume.

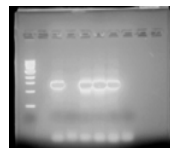


Figure 4: 1% Agarose Gel of PCR Products run for 30 minutes. Lane one is a 1kb DNA ladder; lane two is empty; lanes 3-8 are samples 1-6 respectively (F is no-DNA control). Sample A, C, D, and E each display one band that appear to be equally bright.

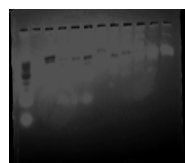


Figure 5: pNIC-Bsa4 with possible PTP1B insert (run in 1% Agarose Gel for 30 minutes). This is a repeated, more reliable gel run of Figure 2. The gamma and contrast has been increased to ensure clarity of bands causing the “black on white” appearance. Lane one is a 1kb DNA ladder and lanes 2-11 represent Samples 1-9 respectively. There was no more pNIC-Bsa4 insert planned so it was not included as a control. Samples 1, 4, 5, 6, 7, 8, and 9 all have three bands. Sample 2 appears to have 4 bands and Sample 3 appears to have one band.

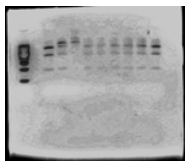


Figure 6: pNIC-Bsa4 with possible PTP1B insert (run in 1% Agarose Gel for 30 minutes). This is a repeated, more reliable gel run of Figure 2. The gamma and contrast has been increased to ensure clarity of bands causing the “black on white” appearance. Lane one is a 1kb DNA ladder and lanes 2-11 represent Samples 1-9 respectively. There was no more pNIC-Bsa4 insert planned so it was not included as a control. Samples 1, 4, 5, 6, 7, 8, and 9 all have three bands. Sample 2 appears to have 4 bands and Sample 3 appears to have one band.

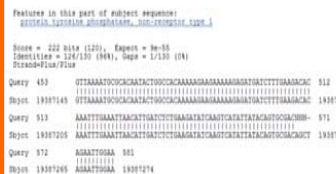
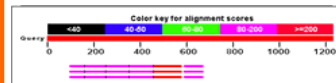


Figure 7: The figure below displays the alignment score and a small portion of the alignment. The data was obtained by using the sequence (forward primer) obtained from diforce at UT Austin and BLASTed against the Human Genome on the NCBI website. As can be seen by the sequence was most closely matched to Protein Tyrosine Phosphatase N1 (PTP1N). The majority of the sequence shows a “high” level of alignment and a smaller portion shows a “very high” level alignment.

RESULTS

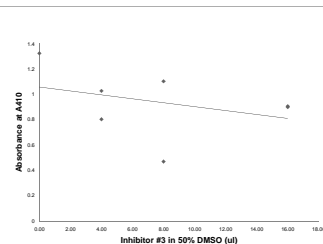
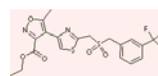


Figure 1: The absorbance of enzyme substrate complex in the presence of varying concentrations of Inhibitor #3. A negative slope is seen here verifying slight inhibition.



Compound: Inhibitor #3
IUPAC name: ethyl 5-methyl-4-[(2-[(3-(trifluoromethyl)benzyl)amino]methyl)methyl]-1,3-thiazol-4-yl]isoxazole-3-carboxylate
Gold Score: -

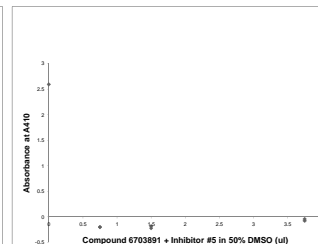
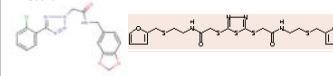


Figure 2: The absorbance of the enzyme substrate complex in the presence of varying concentrations of ‘cocktail’ (combination of compound 6703891 and Inhibitor #5). An extreme negative effect of cocktail is seen.



Compound: 6703891
IUPAC name: N-(1,3-benzodioxol-5-yl)methyl-2-[5-(2-chlorophenyl)-2H-tetrazol-2-yl]acetamide
Gold Score: 64.8447

Compound: Inhibitor #5
IUPAC name: N-[(2-[(2-furylmethyl)thio]ethyl)-2-[5-[(2-[4-(2-furylmethyl)thio]ethyl)amino]-2-methoxy]phenyl)-1,3,4-thiadiazol-2-yl]acetamide

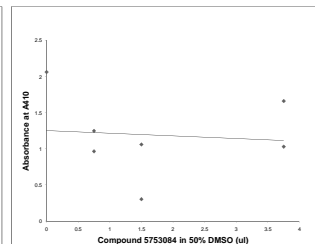
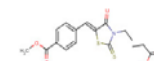


Figure 3: The absorbance of enzyme substrate complex in the presence of varying concentrations of compound 5753084. A slight negative effect is seen.



Compound: 5753084
IUPAC name: 4-[(5-[4-(methoxycarbonyl)benzyl]idene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]butanoic acid
Gold Score: 83.8

DISCUSSION & CONCLUSIONS

Based on GOLD virtual screening three compounds were chosen as possible inhibitors to be tested in wet lab. One compound (6703891) was mixed with an unlikely inhibitor (inhibitor #5). Of the candidates this ‘cocktail’ showed the most inhibition. With only 0.75 microliters of a 50mM solution apparent complete inhibition was seen. It is unknown which compound, if not both, are inhibiting the protein. In testing of Inhibitor #3 and compound 5753084 only slight inhibition was seen.

The cocktail was diluted to 50mM in DMSO. Therefore DMSO had to be tested independently to determine if it was causing any inhibition. In the DMSO only assay, DMSO showed no apparent ability to inhibit formation of the PTP1B/pNPP complex.

FUTURE DIRECTIONS

Research thus far has produced a promising candidate for PTP1B inhibition: N-(1,3-benzodioxol-5-ylmethyl)-2-[5-(2-chlorophenyl)-2H-tetrazol-2-yl]acetamide. However, this inhibitor was mixed with unlikely candidate ‘inhibitor #5’. Since this cocktail showed significant inhibition explorative experimentation would be completed to determine which inhibited or if it was a product of both. Once this is completed the successful inhibitor (if any) would be tested at lower and lower concentrations to determine if it has an IC₅₀ (amount of inhibitor necessary to inhibit half of enzyme) that is low enough to be used as a drug.

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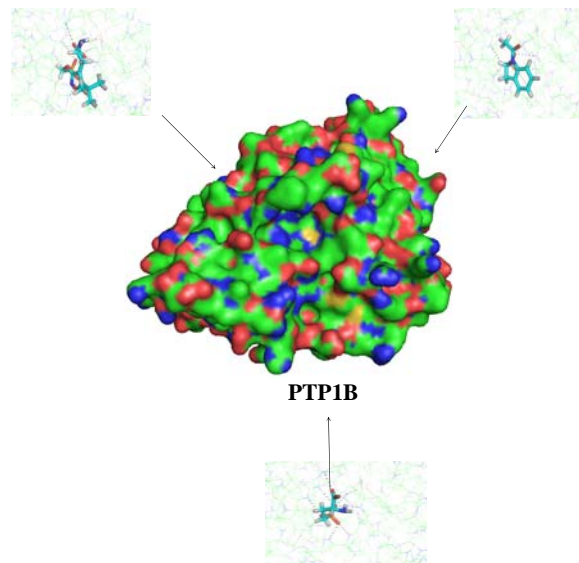


Figure 8: The PTP1B enzyme (surface view) surrounded by possible ligand inhibitors.